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Report

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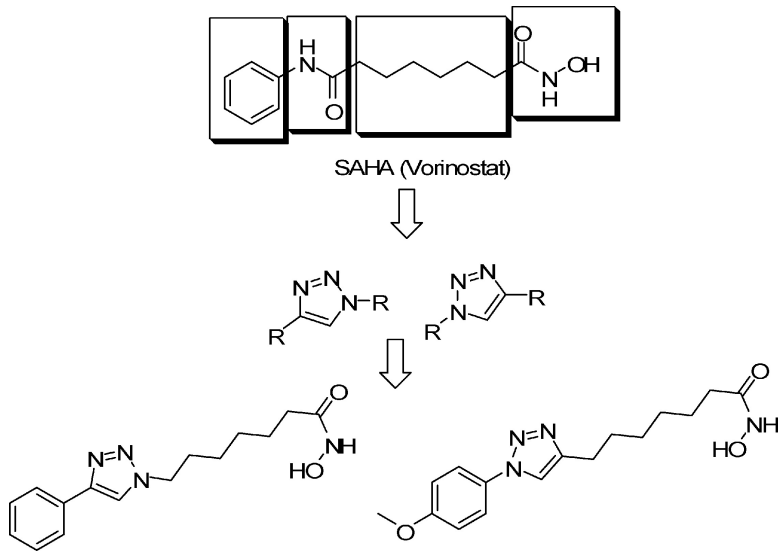

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Triazole-Modified Histone Deacetylase Inhibitors As a Rapid Route to Drug Discovery

Tracey Pirali,[†] Francesca Pagliai,[†] Ciro Mercurio,[‡] Roberto Boggio,[‡] Pier Luigi Canonico,[†] Giovanni Sorba,[†] Gian Cesare Tron,*[,†] and Armando A. Genazzani[†]

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Histone deacetylases (HDACs) are promising targets for a number of diseases, including cancer, as they provide a means to alter transcriptional (i.e., histone acetylation state) and post-transcriptional (i.e., nonhistone proteins acetylation state) regulation in a number of different settings.[1–3] Since the discovery of the differentiating effect of DMSO on erythroleukemia cells, a great effort has been placed in developing new drugs that inhibit histone deacetylases and in elucidating their mechanism of action,[4,5] and the first drug in this class has now been launched on the market (SAHA, Vorinostat, **1**) (Figure 1),[6] while others are in clinical trials.[7] The medicinal chemistry of HDAC inhibitors is flourishing with a great number of reports of active molecules.[7,8] Yet, this is not to say that the identification of novel chemotypes would not be of importance, as potency and specificity on the different subtypes is an important task that still needs to be addressed. Indeed, it is likely that the advent of a novel generation of ligands will not only allow for a better treatment of tumors, but also of other diseases which have been postulated to be sensitive to these drugs (e.g., neurodegeneration, inflammation, etc).[2]

It has been proposed that a triazole moiety can act as a non classical bioisostere of amides.[9,10] In the present contribution, we decided to investigate whether the amide on SAHA could have been replaced with a triazole without a significant loss of activity. This would provide a rewarding strategy to design novel SAHA analogues via a combinatorial approach, as click chemistry could be employed. On the basis of these assumptions, we decided to employ the [3 + 2] azide-alkyne cycloaddition catalyzed by copper (I) salts, the classical click chemistry reaction.[11] If this strategy were to work for HDAC inhibitors, the simplicity of this reaction and its regioselectivity, coupled to the ease of purification of the products, would make it possible to generate with small effort large libraries exploring the chemical space.

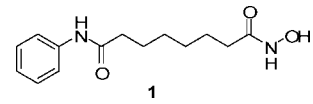

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Figure 1. Structure of SAHA.

We now report that click-SAHA compounds are active and display potencies, both as cytotoxicity and enzyme inhibition, in the same order of magnitude as its parent compound. As a proof of principle, we also decided to change by combinatorial click chemistry the hydrophobic cap group and found that at least one compound was more potent than SAHA. This now opens the avenue both to the generation of novel chemotypes and to the identification of the best cap group/linker/Zn-binding group combinations via combinatorial click chemistry.

We first synthesized click SAHA analogues, using three hydrocarburic spacers of different lengths (Figure 2). The terminal alkyne derivatives bearing the hydroxamic acid were prepared starting from the commercially available alcohols with an internal alkyne through the acetylene-zipper reaction as reported in the literature.[12] Oxidation of the alcohols using Jones reagent afforded the corresponding carboxylic acids that were then converted to the hydroxamic acids using the mixed anhydride method (ethyl chloroformate, *N*-methylmorpholine, and then freshly prepared hydroxylamine; see the Supporting Information for a full characterization of these compounds).[13] Using the same protocol, the terminal azide derivatives bearing the hydroxamic acid moiety were prepared. Then, the phenylazide or the phenylacetylene was reacted with the corresponding hydroxamic acid derivatives in the presence of catalytic amount of copper sulfate (0.05 equiv) and sodium ascorbate (0.1 equiv) in *tert*-butanol-water. The reaction was heated at 60 °C for 24 h, and the resulting triazole derivatives were obtained through simple filtration. It is remarkable that the hydroxamic group, a chelating moiety, does not interfere with the reaction. Yet, this is in accord with previous reports on the synthesis of metalloprotease inhibitors bearing a hydroxamic group.[14] This procedure led to the synthesis of 6 analogues, three of which bear the triazole as a true nonclassical isostere of the amide as represented in SAHA (**5**−**7**; Figure 2), while three bear the structural isomer (**2**−**4**; Figure 2), which might mimic the inverse amide (with the carbonyl group of the amide linked to the phenyl group).

To test whether the compounds synthesized in this manner (**2**−**7**) were active, we decided to employ a cytotoxicity assay on a neuroblastoma cell line (SH-SY5Y).[15] Cells were incubated for 48 h with increasing concentrations of compounds and viability was assayed by MTT (Figure 3). Similar data was obtained using a cell count assay, demonstrating that a decrease in absorbance was not due to technical artifacts (data not shown). Furthermore, as it might have been possible that a minute concentration of copper salts might have precipitated with the products, we evaluated also the cytotoxicity of this salt. Neither copper (II) sulfate nor copper (I) iodide displayed significant cytotoxic effects up to 10 μM

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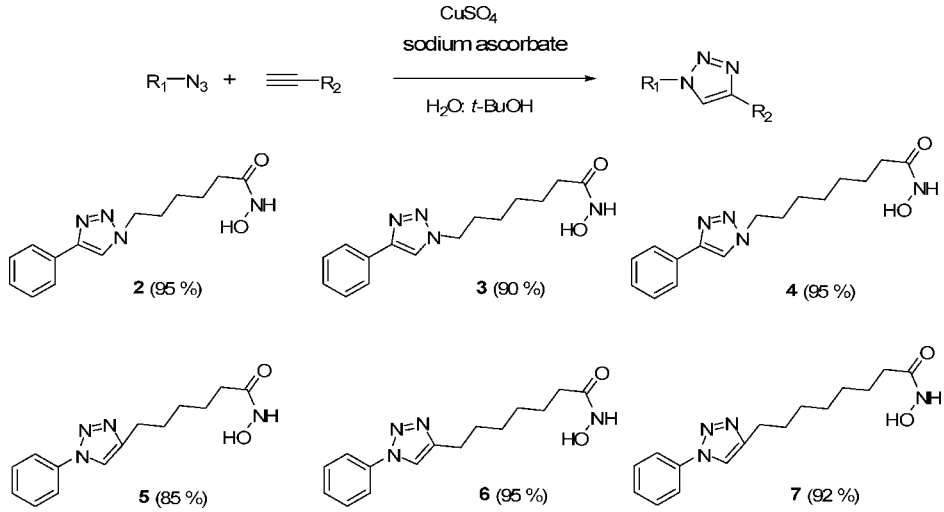


Figure 2. Structure of the triazole-containing SAHA analogues bearing the phenyl cap group.

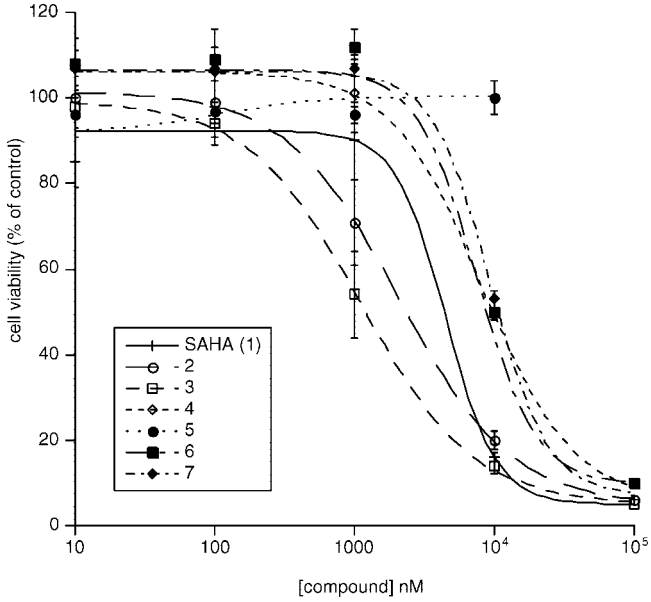


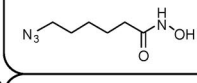
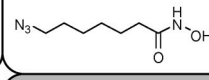
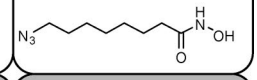
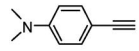
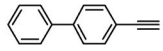
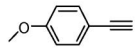
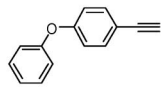
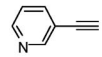
Figure 3. Concentration response curves of SAHA and of SAHA analogues bearing the phenyl cap group. Values represent mean ± SEM of at least eight determinations from two independent experiments on a neuroblastoma cell line.

(percent viability at 10 μM compared to control: 100.5 + 7.9% and 99.1 + 4.4%, respectively). In this cell model, SAHA displayed an IC_{50} for cytotoxicity of approximately 4.5 ± 2.0 μM, which is compatible with what reported by others on cell culture models under similar conditions.[16] Of the 6 novel compounds tested, **2** and **3** displayed an approximately IC_{50} lower than the reference drug (2.1 + 0.1 and 1.1 + 0.06 μM, respectively) while **4**, **6**, and **7** displayed IC_{50} values close to 10 μM. Compound **5** was devoid of activity up to 10 μM, although it was cytotoxic at higher concentrations. It is surprising that all compounds were active, albeit at different extents, as the two structural isomers of the triazole ring should mimic the amide on SAHA or its inverse, and the inverse amide analogue of SAHA had been previously shown to lose most of its activity.[16] The hydroxamic group on all of these compounds would suggest that these molecules act as HDAC inhibitors, but we decided to validate this statement performing preliminary enzymatic assays at a fixed 1 μM concentration of inhibitors **2** and **3**. Indeed, both appeared to be more active than SAHA in this assay, with a reduction down to 22% + 4% and 16% + 3% of control activity, compared to SAHA (43% + 5.4%), which was slightly less efficacious.

As our compounds appeared to be active and acting on histone deacetylases, this prompted us to investigate whether parallel combinatorial chemistry (similar to that reported previously on click-resveratrol)[17] was applicable. We decided to use different hydrophobic cap groups joined by different length linkers to the hydroxamic acid moiety (Tables 1 and 2). The cap groups were chosen randomly, and both triazole structural isomers were investigated. Of the 30 reactions, 29 resulted in a precipitate. The reactions was filtrated and the precipitate was washed with water and diethylether and the solid was submitted to mass analysis to confirm the exact nature of the desired product. The 29 confirmed unpurified compounds were tested on a leukemia cell line (K562), as this is more amenable to rapid screening. Also in this setting, SAHA displayed an IC_{50} for cytotoxicity between 1 and 10 μM. It is interesting to note that of all compounds tested, a few were inactive up to 10 μM, while most were as active as SAHA. One compound, **Eh**, appeared to have a higher cytotoxic potential on K562. From a general perspective, the screening suggested that C7 and C8 linkers were more active compared to C6. Preliminary random screening of a few active compounds at a concentration of 1 μM (data not shown) and the presence of the hydroxamic acid suggested that these compounds were also active on HDACs.

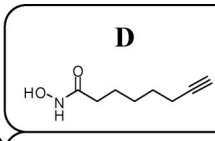
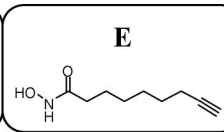
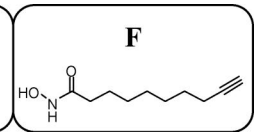
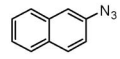
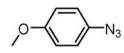
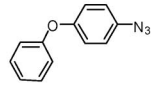
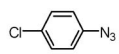
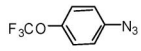
To confirm the data presented above and to further validate our conclusions, we decided to characterize further the two most potent triazoles that emerged from the screening (**Eh** and **Eg**), compound **3** and compound **5**, the latter having displayed only modest activity (Figure 3). The four compounds were purified by column chromatography and tested on three separate cell lines (a colon carcinoma cell line, HCT116; a lung carcinoma, A549; and a human erythroleuemia cell line, K562). Then, the compounds were further analyzed for enzyme inhibition in a biochemical and a cellular model (see the Supporting Information for details; Table 3 for results). All compounds were cytotoxic on the

Table 1. Evaluation of Cytotoxicity in K562 using the Hydrophobic Cap Group Bearing an Alkyne Moiety[a]

	A 	B 	C 
a 	84 ± 7.0 79 ± 6.8	82.0 ± 8.4 19.0 ± 2.5	96.0 ± 10 26.2 ± 2.1
b 	98 ± 8.9 108 ± 8.2	99.3 ± 9.8 58.8 ± 8.7	69.9 ± 4.8 16.7 ± 1.1
c 	81 ± 4.8 25 ± 2.3	83.8 ± 6.5 18.2 ± 1.3	105 ± 7.0 15.5 ± 4.2
d 	No precipitate formed	97.3 ± 7.3 18.7 ± 3.5	92.1 ± 4.5 32.7 ± 2.8
e 	96.3 ± 6.8 111 ± 8.0	81.3 ± 4.0 13.7 ± 0.8	103 ± 6.0 66.1 ± 1.5

[a] For each box, top values represent percent viability at 1 μM and bottom values represent percent viability at 10 μM. Values represent mean ± SEM of at least eight determinations from two separate experiments.

Table 2. Evaluation of Cytotoxicity in K562 using the Hydrophobic Cap Group Bearing an Azide Moiety[a]

	D 	E 	F 
f 	95 ± 6.9 51.4 ± 4.2	86.1 ± 6.7 16.3 ± 0.9	81.3 ± 6.4 61.3 ± 3.3
g 	94.5 ± 6.8 79.0 ± 2.9	66.7 ± 7.1 14.1 ± 0.9	94.6 ± 10.1 62.3 ± 4.1
h 	84.0 ± 4.2 23.5 ± 6.8	44.1 ± 2.6 16.1 ± 0.8	91.4 ± 6.3 53.9 ± 5.4
i 	120 ± 3.8 115 ± 7.9	76.6 ± 6.1 14.2 ± 0.6	98.5 ± 3.6 19.7 ± 0.3
j 	88.2 ± 14.4 64.6 ± 6.5	92.0 ± 8.4 15.3 ± 3.0	81.9 ± 8.0 80.6 ± 3.3

[a] For each box, top values represent percent viability at 1 μM and bottom values represent percent viability at 10 μM. Values represent mean ± SEM of at least eight determinations from two separate experiments.

three cell lines, with **3** and **Eg** displaying similar cytotoxicities compared to SAHA. In the biochemical assay, **3** was about 4-fold more potent than SAHA, and **Eg** was 2-fold more potent, while the other two compounds were less potent once again. A different protocol set to validate the effects in a cellular environment, though, suggested that **Eg** and **3**

Table 3. Biological Evaluation of Selected Compounds[a]

	cytotoxicity (IC_{50}; μM)			enzymatic inhibition (μM)	histone acetylation at 500 nM (% of SAHA)
	HCT116	A549	K562		
SAHA	0.76 ± 0.05	1.08 ± 0.12	0.65 ± 0.11	0.089 ± 0.02	100
3	1.39 ± 0.05	1.98 ± 0.01	1.21 ± 0.2	0.029 ± 0.01	108
5	9.37 ± 0.03	12.5 ± 0.81	8.35 ± 0.56	0.182 ± 0.02	48
Eg	1.78 ± 0.2	2.84 ± 0.25	1.53 ± 0.14	0.049 ± 0.01	85
Eh	3.55 ± 0.24	4.34 ± 0.13	3.3 ± 0.67	0.378 ± 0.02	53

[a] Enzymatic inhibition was evaluated with a commercial in vitro kit (Biomol). Values on the histone acetylation column are referred to the ability of SAHA to increase acetylated histone levels in a cellular environment. At 5 μM, all compounds induced similar histone acetylation levels compared to SAHA. For methods, see the Supporting Information.

had similar histone acetylation effects compared to SAHA (all at a concentration of 500 nM).

In conclusion, we have shown that click chemistry is applicable for the synthesis and screening of novel SAHA-like compounds acting on HDACs. Indeed, we have synthesized and tested in vitro compounds which have a higher affinity for the enzyme, as determined by biochemical enzymatic assays (i.e., **Eg** and **3**).

As a proof of principle, we opted for very simple cap groups (some of which already exploited for amide-bearing SAHA analogues),[16] but it is important to stress that click chemistry is tolerant of almost all functional groups, and therefore the potential combinatorial library resulting from this approach could really explore the chemical space for HDAC inhibitors. On the contrary, amidation reactions require expensive coupling reagents and in some circumstances the protection of functional groups, although this procedure has been used successfully in the rapid discovery of inhibitors.[18] These facts give click chemistry an obvious advantage that deserves to be exploited in the HDAC field. Indeed, we are in the process to investigate the role of the triazole on SAHA by moving the triazole along the hydrophobic linker.

Last, during the submission process of this manuscript, we became aware that another group has synthesized active triazole-based HDAC inhibitors via a different synthetic approach, and their biological conclusions are in accord to those published here.[19]

Acknowledgment. Financial support from Università del Piemonte Orientale and Regione Piemonte (Ricerca Applicata 2004 to AAG) is gratefully acknowledged.

Supporting Information Available. Synthesis of all compounds and their characterization (MS and ^{1}H and ^{13}C NMR data), elemental analysis of the target compounds, and methods of the biological section. This material is available free of charge via the Internet at http://pubs.acs.org.

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